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14. ABSTRACT

The project studies the role of Hedgehog/Gli Signaling in generating the androgen-independent growth phenotype of castration resistant prostate cancer and will test the ability of drugs that target Hedgehog/Gli as a means to suppress the androgen independent growth behavior associated with castration resistant prostate cancer. The work has 3 Aims and work will determine the specific roles for Hedgehog signaling molecules, Smo and Gli in androgen independent growth behavior; work in Aim 2 will characterize the interaction between the androgen receptor (AR) and Gli2 to identify mechanisms of Gli support of AR activity in an androgen-free microenvironment; work in Aim 3 will determine the extent to which Gli activity is involved in intratumoral steroidogenesis that is believed to drive resistance to castration- and other hormonal therapies. The collective goal of this project is to understand the basis for Hedgehog/Gli support of castration-resistant prostate cancer and to identify the appropriate small chemicals that target the signaling network as a prelude to validating new, more effective treatments for castration-resistant prostate cancer.

ANNUAL REPORT

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INTRODUCTION

The project addresses the clinical conundrum faced by the failure of androgen deprivation therapy to control advanced (metastatic) prostate cancer (PCa) for a suitable length of time and the eventual progression of the disease in hormone-treated patients to “castration resistance” that is the overt source of mortality from PCa. The hypothesis that was addressed in this work was that key elements of the hedgehog signaling pathway, particularly the Gli transcription proteins, were participants in the molecular process through which PCa progresses to castration resistance and that, by identifying the most important proteins in the hedgehog signaling pathway that enable castration resistance, one can select target-specific drugs to prevent or block the emergence or continued growth of castration resistant PCa (CRPC). The research report below highlights work done specifically during the last period of the project and then summarizes what was learned over the entire period of the project and how the added knowledge can be used to benefit patients with advanced PCa/CRPC.

BODY

Key Research Accomplishments During this Period

Aim1. Gli2 is a transcription factor that is “activated” by functional hedgehog signaling and this protein is overexpressed in castration resistant PCa tumors (1, 2). While this protein is able to function as an independent transcription factor, we had previously found evidence, based upon co-immunoprecipitation studies, that Gli2 can interact with the androgen receptor (AR) protein and that this interaction allowed the AR to function in PCa cells in the absence of AR ligands (2). In essence, we proposed that Gli2 is an androgen-independent AR co-activator. Since hyperactive AR activity (in androgen-deprived patients) is believed to be part of the mechanism associated with the development of CRPC, our goal in Aim1 of the project was to determine: 1) whether Gli2 can bind directly to AR (or whether the association between Gli2 and AR was mediated by a secondary protein); 2) to identify the precise polypeptide binding domains through which Gli2 and AR interact and; 3) to determine the regions of Gli2 that are required for androgen-independent AR co-activation. We have now accomplished all of these goals and our outcomes have

- A. The AR binding domain within Gli2 is now identified and confirmed. In our previous report, we described our evidence supporting direct binding between AR and Gli2 (using the GST-pulldown method) and we proposed that there were two separate polypeptide binding domains within the C-terminus of the Gli2 protein that could directly bind to AR protein (amino acids [aa] 628-897 and 987-1091). Further followup of this work now only supports the existence of a singular binding domain for AR within the C-terminal domain of Gli2 (aa628-987) as we were not able to reproduce the binding of the aa987-1091 Gli2 peptide in repeat experiments (Figure 1, below).
- B. The domains within Gli2 that are necessary for androgen-independent co-activation of AR are now identified and confirmed. Androgen-independent co-activation of AR-mediated transcription by Gli2 requires both the AR binding domain (at aa628-987) as well as the Gli2 activation domain that lies at the far C-terminus of the Gli2 protein (aa1252-1586) (Figure 2, below). Our result then shows that the AR co-activation by Gli2 is independent of the Gli2 N-terminal or DNA-binding domains. Co-activation was highest using the complete C-terminal of Gli2 (containing up to aa1586) but fragments that contained only portions of the Gli2 activation domain (down to aa1252) also showed more limited AR co-activation activity.

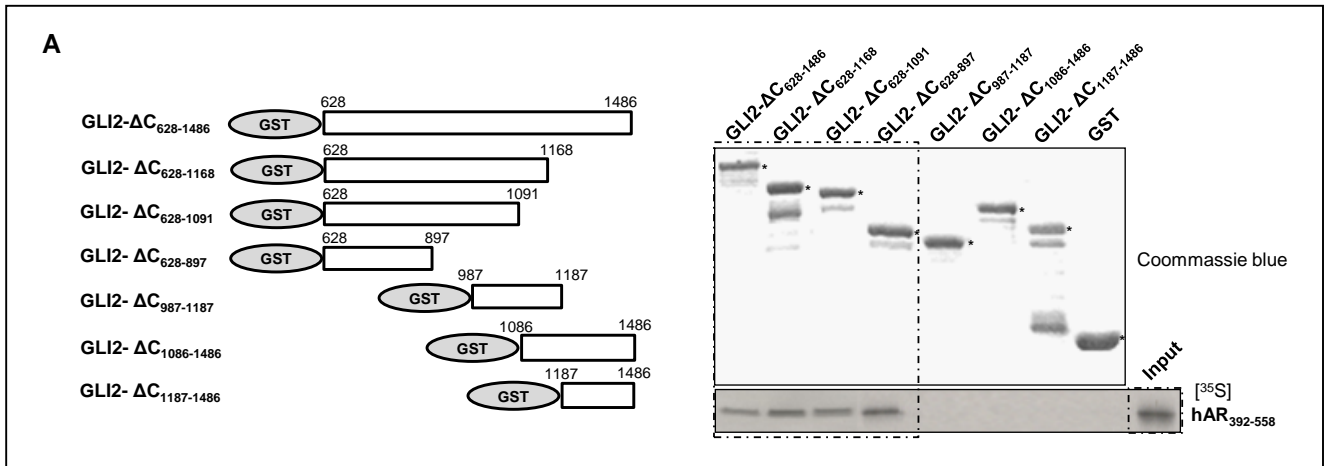


Figure 1. (Left) Limited C-terminal regions of human Gli2 defined by the amino acid numbers indicated were tagged with glutathione s-transferase (GST), inserted into a bacterial expression vector and were used to transform E coli. Peptides made from the transformed bacterium were purified on a glutathione bead column and their presence was confirmed by acrylamide gel electrophoresis (upper right) stained by Coomassie Brilliant Blue. The individual peptides were then mixed with an *in vitro* ³⁵S-labeled peptide fragment of AR (aa352-558) and were incubated. The admixtures were then run over an anti-GST antibody column to re-capture the tagged Gli2 fragments and the column was rinsed extensively before elution in a SDS-containing buffer to release the tagged Gli2 fragment. The eluates were electrophoresed on an acrylamide gel and the gel was exposed to film to show the presence of the captured AR fragment (right bottom). All Gli2 fragments that contained aa628-897 region also eluted with the AR fragment but any Gli2 fragments that did not contain this region did not show binding to the AR fragment.

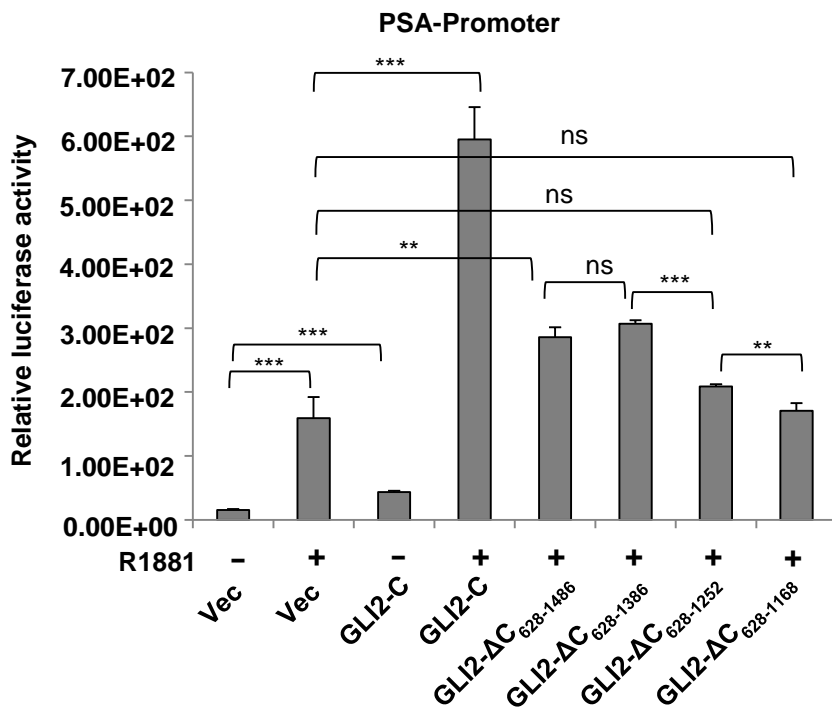


Figure 2. (Left) Co-activation of reporter expression from a PSA enhancer/ promoter luciferase reporter in 293T cells. 293T cells were co-transfected with full length AR and partial Gli2 expression vectors as indicated along with the reporter. Luciferase (luc) expression was measured in a luminometer 24 hrs after transfection. Results show that the complete Gli2 C-terminus (Gli2-C) that includes the entire Gli2 activation domain yields the highest output of luc activity. Gli2 C-terminal fragments that lacked the aa628-897 AR binding domain had no co-activation effects on luc expression (data not shown)

C. The specific polypeptide domain within the AR that binds to Gli2 is now identified and confirmed.

In our report from the last period we showed that either the entire N-terminal domain or a portion of the N-terminal domain within AR referred to as tau5 or AF5 (aa352 to 558) (3, 4) recognizes and binds to the Gli2 C-terminal domain (aa628-897). During this period, we reconfirmed this binding and also showed that neither the AR DNA Binding Domain (aa556-656) nor the AR Ligand Binding Domain has any binding activity with the Gli2 C-terminal domain (aa628-1486) (Figure 3-5). Finally, we showed that a mutation introduced into the critical WxxLF motif (aa435-439) of the AR tau5/AF5 (5) drastically diminished binding of Gli2 (Figure 6, below). Our work then identifies the tau5/AF5 domain as the singular binding site for Gli2 within the AR. Since the tau5/AF5 region is referred to as the androgen-independent activation domain of AR, this identification strongly supports our contention that Gli2 is an androgen-independent co-activator of AR.

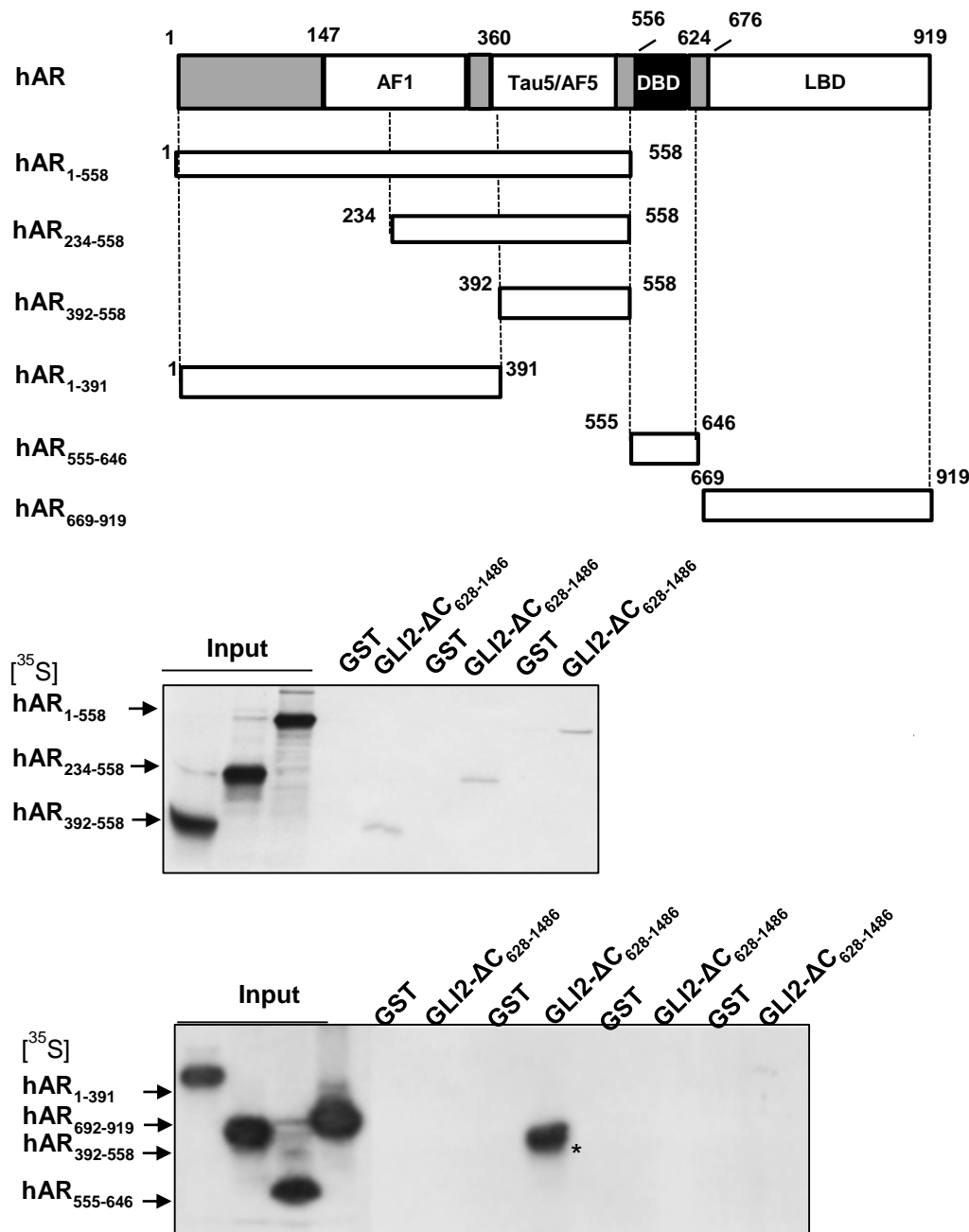


Figure 3. Identification of the Gli2 binding domain on AR. (Upper) AR fragments tested for Gli2 binding activity in the GST-pulldown assay. (Middle and Lower Panels) Autoradiograms of polyacrylamide gels containing eluants of ³⁵S-labeled AR fragments (as indicated) retained on glutathione bead columns after co-incubation with the GST-tagged Gli2 C-terminal domain (aa628-1486). Note that the GST lanes are controls showing retention of ³⁵S-AR fragments without co-incubation with GST-tagged Gli2 C-terminus peptide. Outcomes show that the AR tau5/AF5 domain (aa392-558) is required for AR fragment binding to Gli2.

B

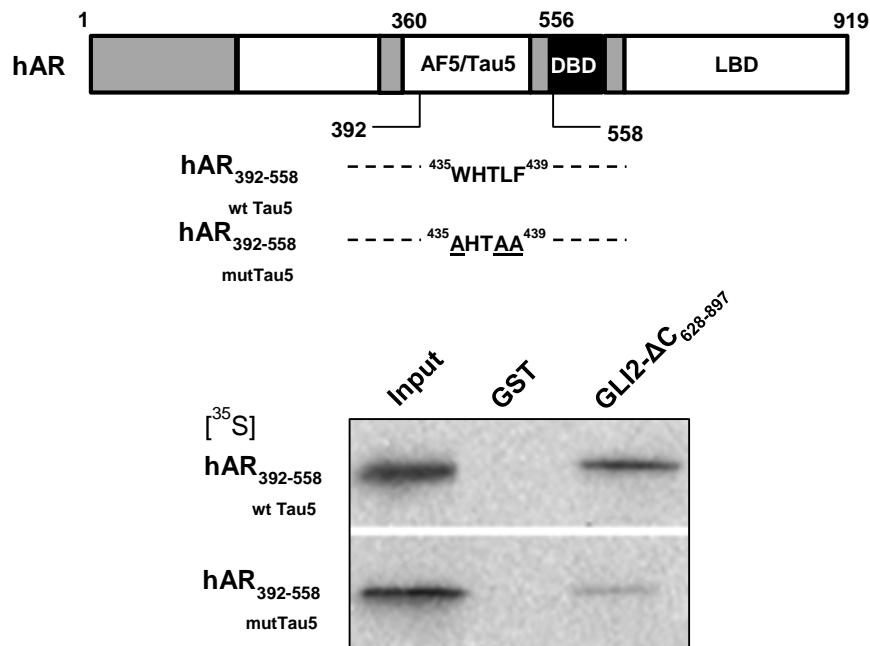


Figure 4. Mutation in the WxxLF motif of AR tau5/AF5 (aa435-439) reduces Gli2 binding. (Top) *In situ* mutagenesis was used to change the WHTLF motif in AR tau5 to AHTAA as previously described. (Bottom) Binding to the GST-labeled Gli2 (aa628 to 897) was significantly reduced by the mutation (lower panel) compared to wildtype tau5/AF5 (upper panel).

D. Gli2/Gli2 C-terminal domain is also a unique co-activator of naturally truncated ARs (AR splice variants) that are thought to be associated with the development of CRPC. Recently, we learned that PCa cells can express naturally truncated splice variants of AR that lack the C-terminal ligand binding domain (6, 7). Increasing evidence suggests that these truncated ARs have an important role in the development and continued growth of CRPC and that they also play a role in resistance to the new generation anti-androgens (such as enzalutamide or abiraterone) (8). These splice variant ARs are thought to have constitutive transcriptional activity that supports AR signaling in patients treated with androgen deprivation therapies/androgen signaling inhibitors. Since we have shown that the co-activation of AR by Gli2 requires Gli2 binding at the tau5/AF5 domain that is located within the common N-terminal domain of all forms of AR, we sought to determine whether Gli2 is also a co-activator of truncated receptors. As is shown in Figure 7, below, Gli2 is indeed a co-activator of the truncation variant AR-V7 so this work establishes that Gli2 overexpression has the potential to effect androgen-independent growth/CRPC driven by truncated ARs as well as by full-length AR.

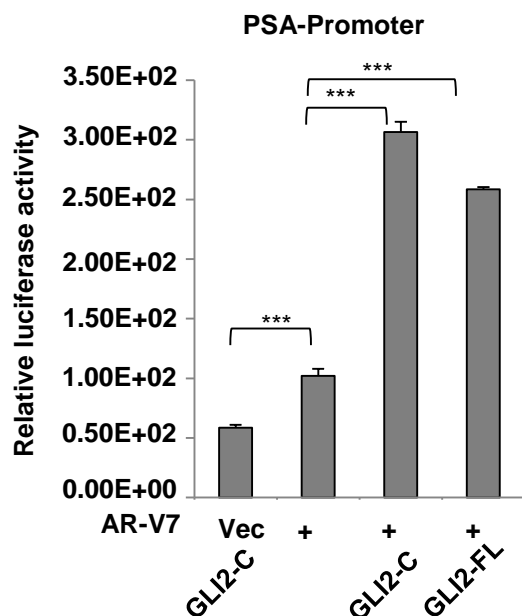


Figure 5. The C-terminal (Gli2-C) or full-length Gli2 (Gli2-FL) co-activates expression of luciferase from the PSA enhancer/promoter reporter vector. Empty vector (Vec) or AR-V7 expression vector was co-transfected into 293-T cells along with Gli2-C or Gli2-FL along with the PSA enhancer/promoter luc reporter. 24 hrs later, relative luc expression was measured. Outcomes show that both Gli2-C and Gli2-FL significantly increase the luciferase expression in cell extracts.

E. Gli2 binds to active androgen response elements (AREs) in the PSA enhancer or PGC promoter of prostate cancer cells *in vivo*. Our outcomes have confirmed a direct binding interaction between Gli2 and AR both *in vitro* and *in vivo*. Other data shows that, when Gli2 or C-terminal fragments of Gli2, are expressed in PCa and other types of cells, they appear to support the transcriptional activity of the AR by acting as an AR co-activator. One final confirmation of this AR co-activator function of Gli2 is our finding that Gli2 accumulates at androgen response elements (AREs) on the chromatin of PCa cells *in vivo*. Here we performed a semi-quantitative ChIP assay that measured pull-down (by immunoprecipitation) of chromatin regions containing active AREs in LNCaP cells that overexpress exogenous myc-tagged Gli2 under a conditional (doxycycline-inducible) promoter (Figure 6 below). Upregulation of Gli2 expression in these cells by doxycycline treatment significantly increased the pulldown of two different AREs, one located in the PSA gene enhancer region and the other located within the PGC gene promoter region (Figure 6).

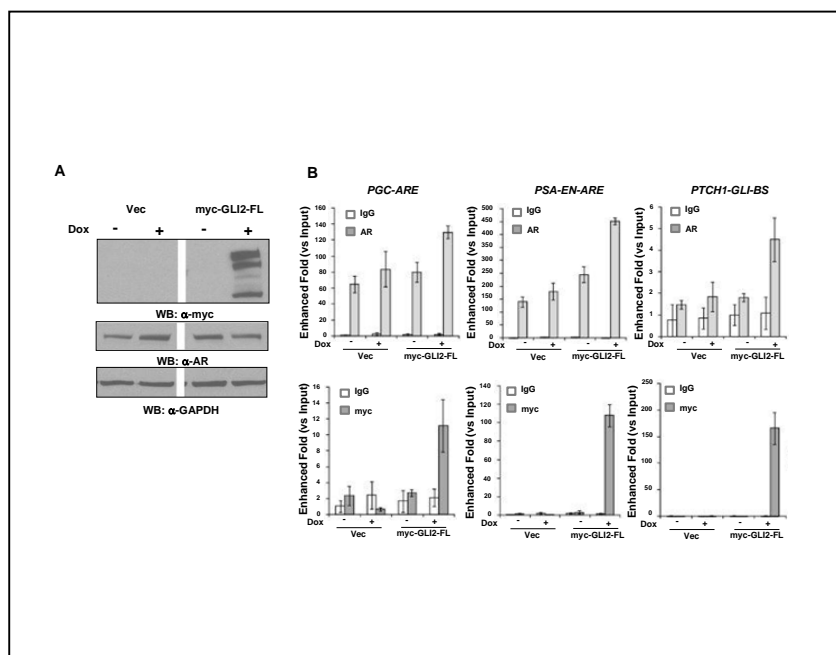


Figure 6. Gli2 is bound to functional AREs in PCa cells. (Left) Western blot shows expression of myc-tagged Gli2 in LNCaP cells under doxycycline. (Right) ChIP with AR or myc antibody shows that anti-myc IP enriches for AREs in the PGC promoter and PSA enhancer whereas anti-AR enriches for GRE in Ptch1 promoter only in cells that overexpress Gli2 under doxycycline.

Aim 2

Progression to CRPC not only involves hyperactivity of the androgen signaling but also a change in the transcriptional program activated by AR (9). This is typified by an *androgen growth-independent variant* of LNCaP cells (LNCaP-AI) that poorly expresses KLK3 (PSA) and KLK2 (traditional androgen-target genes) but instead overexpresses a group of mitotic progression genes under the control of the AR that we refer to as the “Myles Brown genes” (MB genes) because the first report of this alternate transcriptional activity was from the Myles Brown laboratory. We have now performed a series of experiments in the LNCaP-AI cell variant seeking to determine how Gli2 affects the altered AR transcription programs and our results show that Gli2 is not only a co-activator of AR but also appears to play a role in guiding AR to the “alternate” or MB transcriptional program.

A. Gli2 effects on the alternate AR transcriptional program in androgen growth-independent PCa cells.

LNCaP-AI cells were selected by maintenance for over a year in androgen-depleted medium (supplemented with charcoal-stripped FBS). These cells overexpress AR protein (levels approximately 5 times higher than parental LNCaP cells but underexpress KLK3 (PSA) and KLK2 (at about 20% parental LNCaP levels). When LNCaP AI cells were selected for overexpression of exogenous Gli2 (following transduction by a Gli2 expressing lentivirus), their growth rate in androgen-depleted medium increases even further. Coincidentally, overexpression of Gli2 reduces expression of KLK2/3 while it increases expression of two MB genes (so far tested) UBE2C and CDK1 that are associated with androgen-independent growth (Figure 7, below). Finally, treatment of LNCaP-AI cells with the Gli inhibitor drug, GANT61, significantly increased expression of traditional (KLK2/KLK3) AR targets whilst significantly decreasing at least 3 of the MB genes (Figure 8A,B). These outcomes suggest that Gli2 plays a role in re-directing the AR transcriptional program in these cells.

Figure 7 (below). (A) Western blot shows overexpression of myc-tagged Gli2ΔN in LNCaP cells stably transduced with the Gli2 expression vector whereas control cells (transduced with empty lentivector) do not express exogenous Gli2. (B) Real-time qPCR measurement of the expression of KLK2, KLK3 (traditional AR target genes) and UBE2C, CDK1 (MB AR target genes).

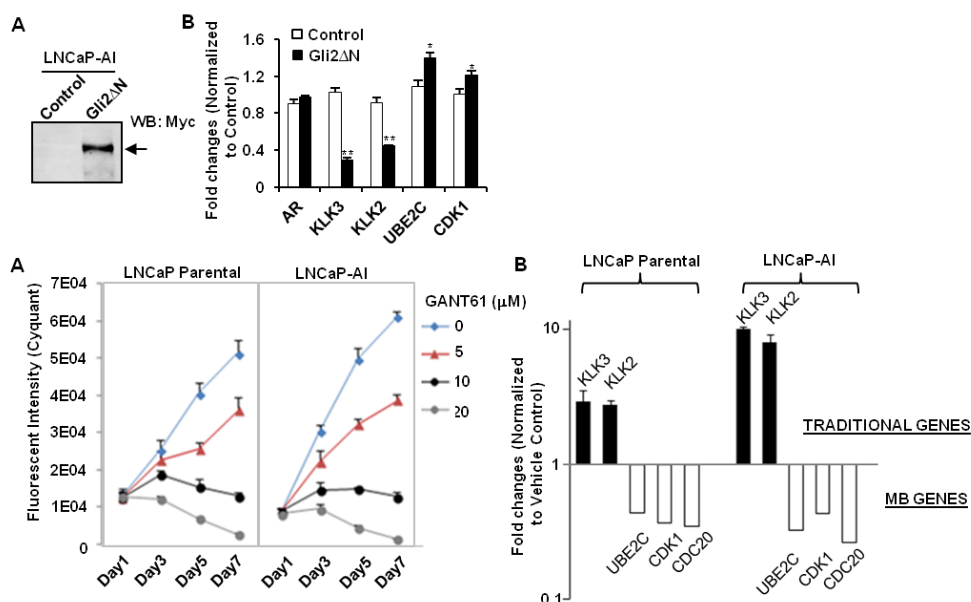


Figure 8 (Above). GANT-61, a Gli-specific inhibitor drug blocks AI cell growth and alters the AR transcription program. (A) GANT61 suppresses LNCaP parental and AI cell growth in a dose-responsive manner. (B) Treatment of LNCaP parental or AI variants with GANT61 at 10 μ M increases expression of traditional AR target genes (KLK2/KLK3) while suppressing expression of alternate AR targets (MB genes represented by UBE2C, CDK1 and CDC20).

Reportable Outcomes During this Period

- We have identified and confirmed a singular binding site for AR within the C-terminus of the Gli2 protein.
- We have shown that the C-terminus of Gli2 is sufficient for co-activation of ligand-independent AR activity and that the activity of this polypeptide requires the AR binding site at the 5' end of the Gli2 C-terminal domain as well as the Gli2 activation domain that is located at the 3' end of the Gli2 C-terminal domain.
- We have identified and confirmed that Gli2 binds specifically at the tau5/AF5 domain of the AR.
- We have shown that the WxxLF motif within tau5/AF5 of the AR is critical for Gli2 binding activity.
- We have shown that Gli2 co-activates naturally truncated (splice variant-generated) AR receptors.
- We have shown functional association of Gli2 with AR on chromatin *in vivo* at cis-acting androgen response elements of androgen-regulated genes.
- We have shown that Gli2 expression affects the AR transcriptional program of PCa cells by enhancing the expression of alternate AR target genes (referred to as MB genes) while suppressing the expression of traditional AR target genes.

Our work during the last year of the project resulted in the presentation of 3 abstracts (below) and an updated abstract will be presented at the 2014.

Li N, Chen M, Buttyan R. Fine mapping of the interaction domains between Gli2 and the androgen receptor. (Presented at the SBUR annual conference, November 2012, Atlanta).

Li N, Chen M, Buttyan R. Identification of the binding domains that enable the ligand-independent co-activation of androgen receptor by Gli2 (Presented at the annual AUA conference, May 2013, Atlanta)

Li N, Chen M, Buttyan R. Identification of the binding domains that allow ligand-independent co-activation of androgen receptor by Gli2 (Presented at the AACR conference, April 2013, Washington DC).

Li N, Troung S, Yu Y, Chen M, Buttyan, R. Gli2 protein and the AR Operational Switch to the Castration Resistant Prostate Cancer Transcription Program. (Will be presented at the AACR conference, April 2014, San Diego).

We are currently completing the assembly of a manuscript entitled: *Determinants of Gli2 Co-Activation of Wildtype and Naturally-Truncated Androgen Receptors* and plan to submit it to *Molecular Endocrinology*. Planned Submission, no later than Feb 1, 2014.

Conclusions

Gli2 is a co-activator of wildtype and truncated androgen receptors that supports acquired growth of prostate cancer cells in an androgen-free environment. Work in the project has defined the critical domains on Gli2 that are required for co-activation of AR; 1) the singular AR binding site that lies at the 5' region of the Gli2 C-terminal domain (aa628-897) and; 2) the extended Gli2 activation domain that lies at the 5' end of the Gli2 C-terminal domain (aa1252-1586). The Gli2 AR binding domain binds to the tau5/AF5 domain within the N-terminal portion of the AR (aa392-558). A mutation within the WxxLF motif found in the AR tau5/AF5 domain significantly suppressed Gli2 interaction with AR so this further establishes the validity of the singular Gli2 binding site (at tau5/AF5) on AR. Furthermore, since Gli2 co-activates ligand-independent activity of AR, this would also be consistent with its binding at tau5/AF5 that is referred to as the "ligand-independent activation domain". Our chromatin immunoprecipitation studies established that Gli2 binds at androgen response elements in prostate cancer cells *in vivo* showing that Gli2 co-activation requires an interaction with AR at the ARE sites. Finally, the nature of the Gli2-AR interaction allows co-activation of a naturally truncated AR variant (V7) that is thought to be active in castration-resistant prostate cancer and we propose that this interaction also contributes to progression to castration resistant disease in prostate cancer patients. Aside from the co-activation effect, we have also found that Gli2 appears to guide AR interaction away from traditional AR targets to an alternate transcriptional pathway characterized by overexpression of genes involved in mitotic progression such as UBE2C and CDK1. The alternate AR transcriptional pathway is dependent upon a chromatin looping mechanism that brings distal AREs into contact with the promoter of the alternate AR gene targets (10) so our study suggests that Gli2 also affects the AR-driven chromatin looping conformation in prostate cancer cells. A drug that targets Gli2 activity, GANT61, suppresses the growth of androgen-dependent and -independent prostate cancer cells by driving the AR away from the alternate transcriptional program back to the traditional AR transcriptional program that is involved in prostate differentiation.

Significance

Gli2, a hedgehog target gene, is known to be overexpressed in castration resistant prostate cancer (11). Our work in the project has now shown that Gli2 overexpression affects two important aspects of androgen signaling that are involved in generation of the castration resistant phenotype of prostate cancer cells; 1) by binding to the androgen receptor protein in a prostate cancer cell, Gli2 activates ligand-independent AR transcriptional activity and; 2) by re-directing the androgen receptor protein to an alternate transcriptional program, it drives the expression of genes that increase the proliferation rate of prostate cancer cells and reduces the expression of genes involved in differentiation of prostate cancer cells. These effects alone identify a unique molecular process through which Gli2 overexpression can effect castration resistant disease. Our observations are validated by our studies using the Gli-inhibitor drug, GANT61 that specifically blocks Gli2 action. GANT-61 not only strongly suppressed the growth of androgen-independent prostate cancer cells but it also redirected the androgen receptor transcription program back to traditional AR target genes (markers of differentiation) and away from the alternate MB transcriptional program. These data suggest that Gli-blocking drugs like GANT61 could have a role in treatment of metastatic and metastatic castration-resistant prostate cancer. In contrast, while we have found that very high doses of smoothened inhibitors (either cyclopamine or GDC-0449) were able to diminish growth of prostate cancer cells, the doses needed for this effect are far in excess of the inhibitory dose of smoothened and the effects on prostate cancer cell growth were relatively modest. Collectively, these outcomes then suggest that the

slight effects of smoothened inhibitors is most likely due to off-target effects that are found at very high concentrations of smoothened inhibitors. In summary, our work suggests that direct Gli inhibition (by Gli inhibitor drugs) appears to be a better strategy to directly prevent/inhibit castration resistant disease compared to smoothened inhibition. So we have achieved the overall goal of our project which was to identify the best strategy for targeting the Hedgehog/Gli pathway to block castration resistant prostate cancer.

Planned Future Work

1. Since Gli activity is associated with normal cell functions, the use of general Gli inhibitors in prostate cancer patients might have adverse effects on normal tissues (i.e. potential for toxicity). Our identification of the Gli-AR interaction domains suggests that an alternate strategy to inhibit Gli support of castration resistant prostate cancer cells without affecting Gli functions in normal tissues may be a small molecule that specifically interferes with this binding interaction. One possibility is the use of a small peptide decoy that would specifically block Gli binding to AR so we have agreed to collaborate with Ganesh Raj at the UT Southwestern Medical Center to develop peptidomimetic strategies to accomplish this.
2. The effects of Gli2 overexpression or GANT61 on the AR transcriptional program in prostate cancer cells suggests that Gli2 is also involved in the complex chromatin looping conformations that are needed for the activation of the alternate AR transcription program in castration-resistant disease. In light of this, it is of interest that Gli protein is known to interact with a specific protein (Med12) in the mediator complex that is required for this chromatin looping activity (12). We intend to further explore how Gli2 might be involved in AR-driven chromatin looping in castration resistant disease.

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